AVIAN INFLUENZA

Public at Last, H5N1 Study Offers Insight Into Virus's Possible Path to Pandemic

Depending on your point of view, the study that appears on page 1534 of this issue of *Science* marks another good week for public health experts trying to protect a vulnerable world from a new influenza pandemic—or for future bioterrorists bent on unleashing one.

The paper, from a laboratory led by virologist Ron Fouchier of Erasmus MC in Rotterdam, the Netherlands, describes how a handful of mutations might give the H5N1 avian influenza virus, which typically infects birds, the potential to move easily between mammals and touch off a human flu pandemic. It appears after more than 8 months of often fierce international debate over whether the results should be made public—and whether researchers should have conducted the experiments at all.

Late last year, the U.S. National Science Advisory Board for Biosecurity (NSABB) unanimously asked *Science* not to publish the study's details. (The journal agreed, in principle.) But in March, the same board voted 12 to six in favor of full publication after reviewing a revised and extended version of the manuscript and other evidence (*Science*, 6 April, p. 19). Along the way, the debate prompted influenza scientists to self-impose a landmark moratorium on some types of H5N1 research (see p. 1496), the U.S. government to set new controls on taxpayer-funded studies involving potentially dangerous pathogens, and the Dutch government to consider blocking publication by invoking export-control laws.

The paper is the second one in 2 months to suggest that H5N1 has pandemic potential. Last month, *Nature* published a similar study by Yoshihiro Kawaoka of the University of Wisconsin, Madison, and the University of Tokyo that was also caught up in the controversy (*Science*, 4 May, p. 529).

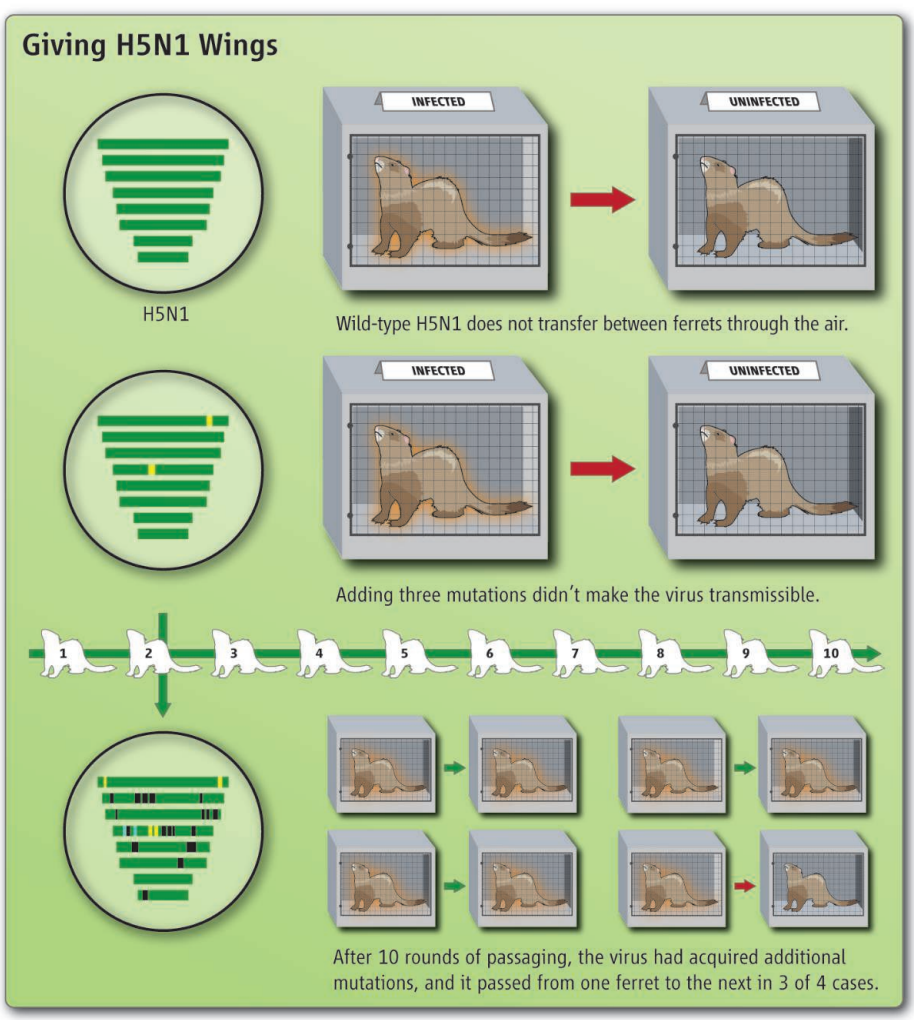
Until now, Fouchier had publicly discussed his study in only very general terms, including in a talk at a September 2011 flu meeting in Malta that triggered wide media coverage. Seeing the data in full is "sobering," says influenza expert Nancy Cox of the U.S. Centers for Disease Control and Prevention in Atlanta, because it suggests that it's easier for H5N1 to trigger a pandemic than other studies—including her own—had indicated. In combination with the Kawaoka paper, Fouchier's findings shed light on how the virus could become pandemic, says Malik Peiris of the University of Hong Kong, and how public health officials might watch for mutations that could send it on its way.

Although it has decimated poultry flocks and killed more than 600 people since it first surfaced in 1997, H5N1 has not touched off a pandemic in humans because it hardly ever spreads from one person to the next—and some scientists think it never will. To become pandemic, the virus would have to become "airborne," or able to spread via tiny droplets spewed out during coughing or sneezing. That is how other influenza strains spread among humans, and both Fouchier and Kawaoka wanted to know which mutations might allow H5N1 to do the same.

There's a key difference between the studies, however. Kawaoka created a hybrid virus: He took the gene for a viral protein called hemagglutinin from an avian H5N1 strain and stitched it together with seven other gene segments from the pandemic H1N1 virus that swept the world in 2009 and 2010, and which is already well-adapted to humans. From this starting point, it took just four mutations in the hemagglutinin gene to create a virus that could travel through the air from one infected ferret—a popular animal model for human infection—and infect another. But Kawaoka's hybrid has not yet been found in nature.

In contrast, "the strong point" of Fouchier's study, Cox says, is that it started out with an actual H5N1 virus isolated from a human victim in Indonesia. In an e-mail to *Science*, Kawaoka agreed that Fouchier's study addresses the most urgent question more directly. "Ron's data are very important," he said.

Fouchier's team first inserted several mutations they knew might help the virus adapt for mammalian spread. One key target was the virus's receptor binding site, the area within the hemagglutinin molecule



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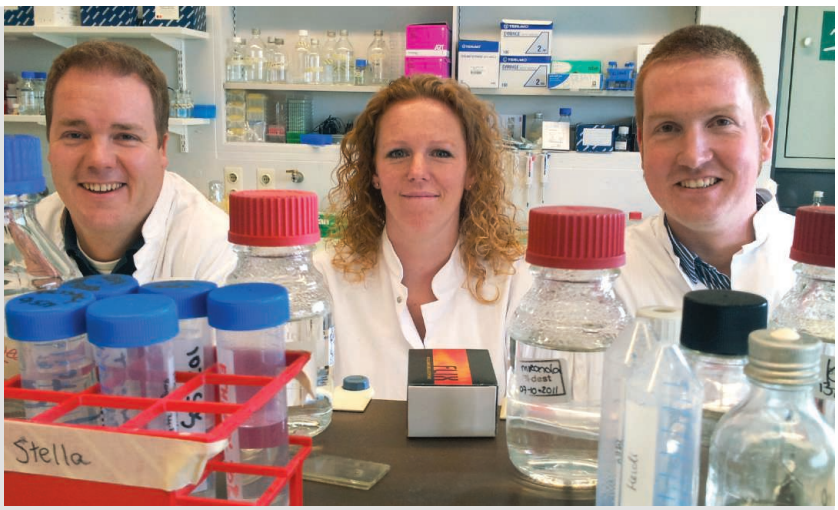
For Young Scientists, A Wild Ride



In the background. Sander Herfst, Eefje Schrauwen, and Martin Linster did the hands-on work in Ron Fouchier's laboratory in the Netherlands.

ROTTERDAM, THE NETHERLANDS—They've become known as the Kawaoka and Fouchier papers. But Yoshihiro Kawaoka and Ron Fouchier rarely set foot in the high-security labs where the experimental work on their two highly controversial H5N1 studies was done. That work—concocting mutant viruses, inoculating ferrets, and testing whether they'd infect others—was carried out by younger researchers who have remained invisible during the past 8 months. Yet for them, the stakes were just as high—higher, perhaps, because a paper in *Science* or *Nature* can be a critical career booster.

Earlier this week, Sander Herfst, a virology postdoc at Erasmus MC here and the first author of Fouchier's paper, published on page 1534 of this issue of *Science*, was getting the champagne ready and announcing a lab party on the bulletin boards. "We're incredibly happy," he says.

While Fouchier gave interviews, traveled to meetings, and lobbied to get his paper published, Herfst stayed in the background, as did Ph.D. students Eefje Schrauwen and Martin Linster, the second and third authors, respectively. Once the National Science Advisory Board for Biosecurity (NSABB) got involved, "it was clear that this was being discussed at a level where we didn't belong," Schrauwen says.

The same was happening at the University of Wisconsin, Madison, where foot soldiers in Kawaoka's lab spent months waiting and worrying whether their paper would ever get published, Masaki Imai, the first author, wrote in an e-mail to *Science*. (*Nature* finally published it last month.) Imai, who is Japanese, obtained a Ph.D. at the University of Hokkaido in Japan, as did the second and third authors, Tokiko Watanabe and Masato Hatta.

In both studies, it was these bench scientists who saw the first signs that they had created new strains of the H5N1 virus that were transmissible from one ferret to another through sneezing and coughing—a finding they realized would be huge news. In Rotterdam, it happened in late June 2011, when a PCR test suggested that a ferret housed in a cage adjacent to an infected one had traces of the H5N1 virus in its airways. "We were very excited," Herfst says. "When we showed it to Ron, he just said: 'Calm down, and do it again. It may be an error.' "

It wasn't. But while he expected to make headlines, Herfst says he never imagined that the paper would get a red light from the NSABB and become the focus of a heated international debate about the limits of academic freedom. Watching the flood of news coverage on TV, "it was strange to think that we had created all of that in our lab," Schrauwen says. "I thought that people would understand how important this kind of work is," Watanabe wrote.

The issue dominated lunch breaks at the lab but began to surface in private conversations, as well. A friend who had read the news stories but didn't know Herfst was involved warned him to watch out "because they are doing some pretty dangerous things at Erasmus." Others asked critical questions: Was this study really necessary? Linster says he could usually convince them. "Debates about animal experiments are more difficult," he says.

Members of both teams, however, worry that the controversy may deter budding scientists from entering the field. "They might be afraid or feel anxious or apprehensive about rejection of papers as a result of biosecurity concerns," Imai wrote. Indeed, a postdoc planning to come to Rotterdam won't work on H5N1, Herfst says. But Hatta thinks it won't be a problem. "Seeking the truth is the job of the scientists. I think nothing affects their motivations," he wrote.

Now that both papers are published, Herfst hopes the moratorium on H5N1 transmissibility studies will be lifted soon (see p. 1496). "We have a long list of interesting things we'd like to do," he says. But Herfst and his colleagues realize that that debate, too, is held well above their pay grade.

–M.E.

that makes first contact with the host cell; scientists already knew that two mutations there can make the virus prefer mammalian cells over bird cells. Another mutation, in the polymerase protein complex, allows the virus to replicate in the cool environment of the human upper respiratory tract rather than in bird intestines, the much warmer environment where it usually resides.

These initial mutations alone didn't do the trick, however, so Fouchier's team decided to try a time-honored method to encourage a pathogen to adapt to a new host: They passed the virus from ferret to ferret by directly inoculating uninfected animals with nasal samples from infected ones and repeated the procedure a total of 10 times. (In his Malta talk, Fouchier called this a "really stupid" approach, a phrase widely interpreted to mean he regretted it. In fact, he says, he just meant that the technique, called passaging, is a simple one compared to the sophistication of creating targeted mutations. The confusion may have stemmed in part from the fact that the Dutch word for "stupid" can also mean "simple.")

The end result was a virus that could move through the air from one caged ferret to another right next to it; in a first experiment, the virus transmitted from cage to cage in three out of four instances.

Prior to publication, media reports suggested that airborne transmission required five mutations. The reality is more complex. Each of Fouchier's transmissible viruses had at least nine mutations, five of which were shared by all. This core quintet may be sufficient, the team writes, but the big question is whether one or more of the other changes also plays an important role, Peiris says.

Fouchier already knows part of the answer. Once his team achieved transmission in the summer of 2011, the researchers began additional experiments to identify the minimum set of mutations needed to make the virus airborne. But before those experiments were finished, they submitted their manuscript to *Science*, worrying that Kawaoka or other scientists might beat them

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How Much Longer Will Moratorium Last?

When will it end? That's what many influenza researchers want to know about the landmark self-imposed moratorium on certain experiments on the H5N1 avian influenza virus that they agreed to earlier this year. The short answer: Who knows?

Initially, the 39 researchers who announced the moratorium on 20 January said it would last just 60 days (*Science*, 27 January, p. 387). But in February, the loosely organized coalition agreed to an indefinite extension to give experts and the public more time to discuss and address concerns about the safety and wisdom of experiments that could alter H5N1 in ways that make the virus more dangerous to humans. (Other H5N1 research, such as the testing of newly detected strains, continued.)

Now, some of the moratorium's signers are eager for research to resume. But many say they are perplexed about how that decision will be reached and who will decide. "I wish I knew how it was going to be resolved," says virologist Robert Webster of St. Jude Children's Research Hospital in Memphis, Tennessee, a moratorium signer who was deeply involved in the flu papers controversy. "We haven't discussed this," says virologist Yi Guan of the University of Hong Kong.

Some key players, meanwhile, predict it will be months before the standstill ends. "We've still got a lot of homework to do … and some boxes to check" before the moratorium should be lifted, believes Anthony Fauci, the head of the National Institute of Allergy and Infectious Diseases (NIAID), which funded the controversial studies by Ron Fouchier of Erasmus MC in Rotterdam, the Netherlands, and Yoshihiro Kawaoka of the University of Wisconsin, Madison, and the University of Tokyo. Although Fauci isn't a signer of the moratorium, he played an influential role in encouraging Fouchier, Kawaoka, and other leading influenza researchers to organize it.

The researchers reluctantly agreed, driven in part by warnings that governments, reacting to public fears and media reports of "doomsday" viruses, might clamp down on the field if scientists didn't act on their own. Some dubbed the move "Asilomar 2," a reference to the historic 1975 agreement among recombinant DNA researchers that halted experiments in their emerging field until safety guidelines were established.

Before the current moratorium can end, several things have to happen, according to moratorium signers, Fauci, and others:

• The U.S. government must release for public comment a document that explains how universities and private laboratories can help federal funding agencies screen proposed research projects for "dual use research of concern" (DURC) that could be used for good or nefarious purposes. The goal of the new DURC screening program, which was announced in late March and covers 15 "high risk" pathogens including H5N1, is to spot problematic studies before they begin. The document—which is expected to run to nearly 30 pages and will be accompanied by a 100-page backgrounder—could be released "sometime this summer," Fauci says.

• Scientists and funders will need to agree on which lines of H5N1 research are—and are not—worth the risks. Particularly problematic, say Fauci and others, are "gain of function" studies, such as Kawaoka's and Fouchier's, in which researchers create mutant viruses that gain capabili-

to the punch. "Usually when you discover something important, somebody else is discovering it, too," Fouchier says. (He was right: Kawaoka, who says he didn't know about Fouchier's work, had submitted his manuscript less than 2 weeks earlier.) Now, Fouchier declines to discuss the results from the additional experiments, which are on hold as a result of the moratorium.

The published paper shows that the core set of five mutations includes the three that the team introduced themselves and two more that arose during passaging. And the resemblance to what Kawaoka found is "quite remarkable," says James Paulson, a glycobiologist at the Scripps Research Institute in San Diego, California. Both teams found that two mutations at the receptor binding site—one of them identical in the two studies—are important; both discovered an additional mutation that makes hemagglutinin lose a sugar group, which apparently helps make room for the mammalian host cell receptor. Kawaoka also found a mutation in the hemagglutinin's stalk that improves the virus's stability and compensates for the other mutations, Paulson says. "It's tempting to think" that one of Fouchier's mutations plays a similar role—although it's not in the stalk but in another area where three hemagglutinin molecules align to form a so-called trimer.

Cox says she's "surprised" that it didn't take more mutations. In a study with Paulson published online by *Virology* in November, her team also mutated the receptor-binding site, but they had to make several other changes—including slotting in a human-adapted version of another viral gene called *neuraminidase*—to get airborne transmission. That led the authors to conclude that the virus might require "extensive evolution" to become pandemic. Fouchier's paper upsets that reassuring notion.

Both papers will aid surveillance efforts because they suggest which genetic changes to look out for in H5N1, Peiris says. But they also point to a limitation: Several mutations can have the same effect on the virus. "It wouldn't be appropriate to focus just on these mutations," Peiris says. "The virus has different ways to go from A to B." More research is needed to discover how many ways, he says.

In a second paper published this week and co-authored by Kawaoka and Fouchier (p. 1541), a group led by mathematician Derek Smith of the University of Cambridge in the United Kingdom takes a stab at understanding the likelihood of the emergence of a pandemic H5N1 strain. The researchers first combed through surveillance databases to determine whether the mutations identified in the two controversial studies have already appeared in

Nothing to sneeze at. The study tested for airborne transmission of H5N1 by placing infected and uninfected ferrets in adjoining cages.

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At a standstill. Certain experiments with H5N1 are on hold until signers of a self-imposed moratorium, including Yoshihiro Kawaoka (*left*) and Ron Fouchier (*right*), agree to lift it.

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ties—such as mammalian transmission—that naturally occurring versions do not have. A key step in this process could come in late July, when the heads of NIAID-funded influenza laboratories are scheduled to meet in New York City.

• Laboratory safety officials and scientists will need to "at least have a consensus on the level of biocontainment required" for H5N1 studies, says microbiologist Adolfo García-Sastre of Mount Sinai School of Medicine in New York City, a leader of the moratorium. Currently, most H5N1 studies occur in biosafety level 3 (BSL-3) laboratories, but some critics argue that they should be restricted to a small number of higher-containment BSL-4 laboratories.

In the meantime, many signers say the moratorium has already achieved its goal. "The voluntary action … helped calm people's concerns so that discussion could take place," Kawaoka says. And it "provided the time to deal with these issues in some depth," says Thomas Mettenleiter of the Friedrich Loeffler Institute in Griefswald–Insel Riems, Germany, even though "no universal 'solution' was found."

–DAVID MALAKOFF

With reporting by Martin Enserink.

nature. They found that many H5N1 isolates are three—and in a few rare cases, just two—mutations away from Kawaoka's quartet and four from Fouchier's quintet.

They then developed a model of viral evolution to test whether existing viruses, once they happen to infect a mammalian host, might accumulate the missing mutations and become excreted in respiratory droplets, which could start a chain of transmission. The model takes into account a variety of factors, such as the duration of the infection and whether individual mutations by themselves benefit the virus. The conclusion, says first author Colin Russell of the University of Cambridge, is that a virus that is only three mutations away from the full set is "likely" to acquire them and end up in droplets. But the paper can't put a number on that risk; there are too many unknowns.

"You can do a lot of fancy maths, but in the end the probability is hard to pin down," Peiris says. Still, "it's a model of how modeling should be done," says Steven Wolinsky, who studies HIV evolution at Northwestern University in Chicago, Illinois. "They do a very nice job of explaining all the caveats."

The study was presented both at a World Health Organization meeting about the papers in February and during the second NSABB review in March, and it helped convince a majority on the panel that H5N1's risks were real enough to warrant publishing Fouchier's paper, says NSABB acting chair Paul Keim, a microbial geneticist at Northern Arizona University in Flagstaff.

Another factor that swayed the board to support publication, NSABB members say, is that the published version of Fouchier's paper does a much better job of clarifying the lethality of his airborne virus than the first version they read. Fouchier says the draft he submitted to *Science* did not discuss whether his airborne viruses killed the ferrets they infected. But two of the three reviewers asked for additional experiments examining lethality, which Fouchier said took just a week to perform, "so we did them," and added a line to the paper describing the outcome.

That language—combined with Fouchier's remarks in interviews and sometimes hyperbolic press coverage—appeared to suggest that the airborne mutants were extremely lethal, which "greatly alarmed" many NSABB reviewers, says virologist Robert Webster of St. Jude Children's Research Hospital in Memphis, Tennessee, an influenza expert who was asked to advise NSABB on the two papers. In fact, however, none of the ferrets had died from airborne transmission; six ferrets that had the virus squirted directly into their trachea all died. But that outcome is "not very relevant" for evaluating the virus's risk, Fouchier notes, because that's not how humans or animals typically contract flu. He says the manuscript NSABB saw in its first review last year made clear that the different routes of infection led to different outcomes.

But Webster says the results were presented in a way that confused NSABB. He says that the experienced virologists involved in NSABB's discussions—including himself—should have pushed harder to clarify those results and emphasize that lethality in ferrets does not necessarily predict lethality in humans. When the lethality data finally became clearer in the months after the NSABB's initial recommendation, many members say they began to reconsider. In retrospect, Fouchier says, "we should have ignored [*Science*'s] reviewers' request for lethality data," given the confusion that ensued.

The publication of Fouchier's paper isn't likely to be the last word on such issues, however, especially as influenza researchers seek to restart similar studies now stalled by the moratorium. Many, however, are treading cautiously, eager to avoid replaying the drama of the last 8 months. Fouchier, for his part, says he's "sick of all these discussions," and he declined to release his first manuscript to reporters in order to help clarify how the story unfolded, a step Keim says he would support. "I want to move on," Fouchier says. "Maybe in 5 or 10 years' time, when someone writes a book about all of this."

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With reporting by David Malakoff.

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